Handling Tobacco During the Fermentation Process in Cases

Another method is to ferment the tobacco in bulk.

BULLETIN 356

MARCH 1938

Studies on the FERMENTATION OF TOBACCO

1. Microflora of Cured and Fermenting Cigar-leaf Tobacco

THE PENNSYLVANIA STATE COLLEGE
SCHOOL OF AGRICULTURE AND EXPERIMENT STATION
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PENNSYLVANIA AGRICULTURAL EXPERIMENT STATION

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Studies on the Fermentation of Tobacco

1. The Microflora of Cured and Fermenting Cigar-leaf Tobacco

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PROCESSING of tobacco of the cigar-leaf type involves certain periods of fermentation. Reactions of an exothermic nature characterized by oxygen uptake and the evolution of carbon dioxide, ammonia and other volatile substances take place during this process. The course of fermentation appears to be affected profoundly by variations in composition of the leaf which are due to differences in environmental conditions, among which may be mentioned the soil, season and cultural practices on the farm. Great losses to the industry are occasioned by the failure of certain crops to undergo a satisfactory fermentation in the warehouses of the manufacturer.

Although the fermentation of tobacco has been the subject of study for more than 50 years, little exact knowledge exists as to the different factors involved. Improvements in methods employed by the manufacturer have been to a great extent the results of empirical information acquired in the industry rather than the result of scientific study of the problems involved.

Three theories have been advanced to account for the chemical changes occurring during the fermentation process by workers who have studied the problem. The first theory advanced maintained that the reactions were purely of an oxidative character and were not catalyzed by enzymes. That the fermentation of cigar-leaf may be explained in this manner is no longer the belief of those familiar with the process, but the literature of recent years

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on the subject of the fermentation of other types of tobacco implies acceptance of this theory. The second theory advanced ascribed the chemical reactions as due to the activities of microörganisms. Although 50 years have passed since the inception of this theory, the support accorded it in recent years has been vague and somewhat contradictory. The third theory attempts to account for the chemical reactions during fermentation on the basis of catalysis by plant enzymes, and this appears to be accepted by the majority of those engaged in the processing of cigar-leaf tobacco at the present time. Its supporters have vigorously attacked the bacterial theory and left it but few adherents.

The present study was undertaken in an effort to reconcile some of the divergent views of previous workers and determine if possible the nature of the fermentation of cigar-leaf tobacco and the mechanism involved.

REVIEW OF LITERATURE

The belief that the changes occurring in fermenting cigar-leaf tobacco may take place in the absence of enzymatic activity is found frequently in the literature on the subject. This theory was advanced by Nessler *(19)*. Although many workers since that time have hesitated to support this non-biological theory, others have found it—in the face of a lack of sufficient scientific evidence—convenient to do so. An unbiased analysis of the observations of many of the investigators in this field tends to indicate that certain of the chemical reactions taking place in the fermentation process are indeed uncatalyzed by enzymes.

Koller *(12)* inoculated tobacco with a yeast infusion in an effort to hasten the fermentation. Fesca *(5)* compared the tobacco fermentation to that of ensilage. The same year Schloesing *(22)* concluded that the process was somewhat similar to that taking place in fermenting manure. These early suggestions relating to the role of microorganisms in the fermentation of tobacco were followed by the observations of Suchsland *(25)* in 1891, who reported the isolation of bacterial cultures from tobacco undergoing fermentation. He further claimed that these organisms were the principal agents involved and that a certain quality could be imparted by artificial inoculation of tobacco. Within the following 10 years Miciol *(18)*, Davalos *(4)*, Behrens *(1)*, Vernhout *(26)* and Koning *(13)* published in general support of the bacterial theory advanced by Suchsland.

Loew *(14)* in 1899 published the first of a series of three papers in which he vigorously attacked the bacterial theory of Suchsland *(25)* and offered the theory that plant enzymes were wholly responsible. The bitter attack of Loew placed the bacterial theory in ill

repute for a period of 25 years, and the influence of this series of publications may be seen in practically all subsequent publications on the subject. Boeckhout & Ott de Vries *(2)*, Jensen *(8, 9)*, Cohen & Jensen *(3)* and Smirnov *(24)* published in confirmation of the work of Loew, and many other research workers accepted his conclusions and based the results of their own investigations upon his theory. The conclusions of Johnson *(10)* admit the possibility of the plant enzyme mechanism.

In 1925 there appeared two publications, Schmidt *(23)* and Joergensen *(11)*, lending support to the bacterial hypothesis of Suchsland. More recently Johnson *(10)* and Giovannozzi *(6, 7)* have presented evidence suggesting that microörganisms may play an important role in the fermentation of tobacco.

The following publications are deserving of particular consideration in connection with the investigation reported in this paper:

Vernhout *(26)* studied cured tobacco leaves and leaves representing various stages of fermentation. He found two distinct types of bacteria consistently present. These proved to be species of *Bacillus* which, according to the description of the author, would be classified today in the *subtilis–mesentericus–vulgatus* group of this genus. He described these as Bacterium A and Bacterium B, of which A grew at somewhat higher temperatures than did B and was slightly less proteolytic. The description of A more closely conforms to that of the *subtilis–mesentericus–vulgatus* group. The author believed B to be more important in the earlier stages, and A to be the important form in later stages of fermentation.

Schmidt *(23)* investigated the fermentation of German and certain other tobaccos, particularly some which were grown in Ohio. He found that the microflora present on the tobacco passed through selective changes as the fermentation proceeded. The particular types of bacteria present during the fermentation were found to vary with the quality of the tobacco, and different methods of fermentation resulted from different types of organisms. On Ohio tobacco of good quality he found only bacteria of the *subtilis–mesentericus–vulgatus* group, while with some of poorer quality he found various cocci. Members of the genus *Clostridium* were found on tobacco of very poor quality which underwent an unsatisfactory fermentation. Short rods, fluorescent bacteria and large spore-formers of the *mycoides* and *megatherium* types were found on some samples.

Johnson *(10)* reported a study of the fermentation of Wisconsin and other cigar-leaf tobacco. He isolated and studied in pure culture species of fungi of *Aspergillus*, *Penicillium* and*Oöspora*, two unidentified cultures of bacteria and a species of *Staphylococcus*. He treated samples of tobacco with chloroform, mercuric chloride,

acetone, toluene, beta naphthol, silver nitrate and heat in an effort to disinfect the samples. He found that all of these treatments, with the exception of silver nitrate, completely checked thermogenesis. The silver nitrate treatment reduced theromogenic activity. He concluded that in the case of this disinfectant he was able to inhibit bacterial activity without seriously affecting enzymatic activity. In the other cases he concluded that he had destroyed both. He reported that a temperature of 85° C for one hour would completely sterilize the samples. Pure-culture inoculation of such heat-treated tobacco showed that the bacterial species isolated were incapable of inducing thermogenesis although species of fungi induced heating. He concluded that microörganisms, especially fungi, may play a role in the fermentation or processing of certain types of cigar-leaf tobacco although such organisms may not necessarily be essential to fermentation.

The attack of Loew *(14, 15, 16)* upon the bacterial hypothesis of Suchsland has been an important factor in all research on this subject in recent years. From a bacteriological standpoint the data of Loew are far from satisfactory, the conclusions being rather arbitrary considering the data presented. Loew indicted the bacterial theory upon a number of grounds. He maintained that direct microscopic observation of fermenting leaves failed to reveal a sufficient population of microorganisms to account for the profound chemical changes of fermentation. He further claimed that the moisture content was insufficient to permit growth of bacteria, and that the expressed juice of the cured or fermenting leaves would remain sterile. Loew claimed that the cellulose of the leaf would of necessity be vigorously attacked if bacteria were the responsible agents and that evidence of cellulose attack in a satisfactory fermentation was lacking. In order to account for the enzymatic activity which he found to be associated with fermentation, Loew postulated the plant enzyme theory. That this activity is entirely due to plant enzymes has been challenged by the authors in previous publications *(17, 20, 21)*.

TECHNIQUE

For the purpose of this study cured tobacco was obtained from growers and manufacturers of Pennsylvania, Wisconsin and Ohio cigar-leaf, and fermenting and fermented tobacco was obtained from manufacturers engaged in the processing of these crops. Samples rated by manufacturers as good, fair and poor were selected and studies were conducted under laboratory and plant conditions.

Two methods of sampling for bacterial assay were practiced throughout the course of the investigation. One method consisted in cutting 100 sq. cm. of surface area from the leaf, the sample

consisting of two 40-sq. cm. and one 20-sq. cm. portion taken from different locations on the leaf. The other method consisted in the removal of a weighed sample, usually 2 grams, although in some cases larger samples were taken in order to procure a representative sample.

In the preparation of suspensions of the samples for bacterial counts and for determining enzyme activity, two methods were also used throughout the course of the study. In one case the leaf material was dried at room temperature and ground in a sterile mortar. The second method was an analysis of the moist leaf, as follows: The moist sample was placed in a sterile 100-ml. water blank containing several glass beads and allowed to stand for 45 minutes. This was followed by a three-minute period of vigorous shaking. Appropriate dilutions for the assay were made in sterile water blanks at this point. Comparison of the results obtained by the use of the two methods will be found in the discussion of experimental results.

Counts of microorganisms were made by the plate method and in many instances this information was supplemented by direct microscopic counts, using the Petroff-Hauser chamber. Preliminary investigation showed beef-extract-peptone agar, adjusted to a pH of 6.8, to be a suitable substrate for use in making the plate counts of bacteria. Media containing various concentrations of tobacco infusion were investigated but seemed to offer no advantage when compared with the common laboratory media. Several energy and nitrogen sources were introduced in preliminary trials but these additions failed also to improve the substrate. In efforts to detect the presence of gram-negative organisms a suitable concentration of crystal violet was added to the beef-extract-peptone agar medium.

For the purpose of determining the relative numbers of fungi on various samples, dilution plates poured with glucose-peptone-acid-agar, adjusted to a pH of 4.5, proved best adapted.

Various incubation temperatures were resorted to in the determination of bacterial numbers. Parallel plates were poured and incubated at temperatures of 20, 30, 37, 45 and 55° C. Plates were occasionally incubated under anaerobic conditions, using potato tissue to remove the oxygen from the atmosphere of the anaerobic jars. Incubation time varied with temperature; a period of 24 hours was found sufficient for temperatures of 37° C and higher, and periods ranging from two days to four days proved adapted to the lower temperatures. All acid-agar plates were incubated at room temperature and readings made on the third and fifth days.

On a number of occasions a pasteurization treatment was resorted to in an effort to destroy all vegetative forms in the sample and

to stimulate the germination of bacterial spores. This was accomplished by holding the water suspension of the sample at 80° C for 15 minutes. As this treatment invariably resulted in the elimination of all fungi and gram-negative organisms it was assumed that all vegetative forms were destroyed in the procedure.

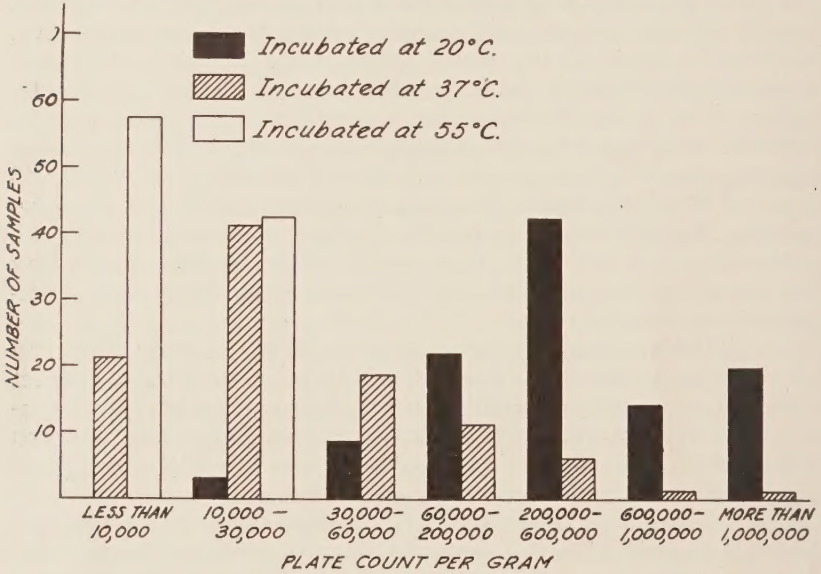

Fig. 1.—Aerobic reproduction of bacteria on nutrient agar incubated at different temperatures, based on studies involving 100 samples of cured cigar-leaf tobacco.

The usual laboratory methods were employed in the pure culture studies of isolations made in the course of the investigation.

Determinations of pH were made colorimetrically or by the use of the glass electrode as noted. Moisture determinations were based on the loss of weight on oven drying at 103° C for a period of four hours.

EXPERIMENTAL RESULTS

Flora of the cured leaf.—During the course of these investigations a great many samples of freshly cured cigar-leaf of the 1936 and 1937 crops of Pennsylvania and Wisconsin tobacco were examined. An analysis of the numbers of bacteria found upon the lamina of 100 typical samples is presented in fig. 1.

Temperature of incubation is an extremely important factor in the determination by plate count of the number of bacteria present. All of the 100 samples showed less than 30,000 viable organisms per gram when the plates were incubated at 55° C. Incubation at

37° C gave somewhat higher counts, while incubation at 20° C gave still higher counts. Approximately 70 per cent of these samples gave values greater than 200,000 per gram at the latter temperature of incubation. No sample examined was found to show less than 15,000 viable bacteria per gram with plate incubation at 20° and none showed less than 6,000 per gram at 37° C. Incubation at 45° resulted in counts greater than those obtained at 55° but

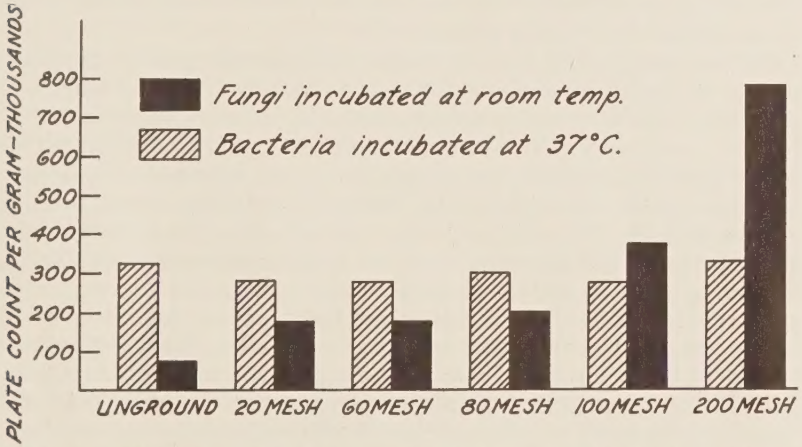

Fig. 2.—Effect of particle size upon the plate counts of bacteria and fungi found on cured cigar-leaf tobacco.

lower than those obtained at 37° C. Likewise counts at 25° and 30° ranged in order between those obtained at 20 and 37° C. Counts at 25, 30 and 45° C are not indicated in fig. 1.

Additions to the standard beef-extract–peptone agar used in preliminary trials and differents methods of sampling failed to show significant differences in counts. In an effort to determine the most suitable method for the preparation of the material under investigation, plates were poured from samples, some of which had been finely ground and others suspended without grinding. The results, typical of the many tried, are shown in fig. 2. No significant effect on the number of bacteria was noted but the effect on the plate count of fungi was marked in every instance, owing to the fragmentation of viable mycelia. Counts of fungi on cured cigar-leaf ranged as a rule between 10,000 and 100,000 per gram when the suspension was prepared without grinding.

Of more significance were the types of organisms found upon the cured leaf. Bacteria present on the plates were largely spore-formers and cocci; fungi present represented for the most part members of *Aspergillus, Penicillium, Mucor* and *Rhizopus*. Many

platings of tobacco leaves revealed the presence of gram negative forms, types that were greatly reduced in number during the curing process. Crystal violet plates inoculated with dilutions of cured tobacco usually were overgrown with fungi, indicating the small numbers of gram negative bacteria surviving the curing process. This is a reflection of the composition of the substrate inasmuch as bright cigarette-leaf tobacco was found to carry a significantly large number of gram-negative rods following the much more drastic flue-curing process.

Having established the fact that a considerable part of the population of the cured cigar-leaf consisted of spore-forming bacteria, the difficulties involved in making reliable determinations of numbers of viable microörganisms became obvious. Plate counts of microörganisms reflect the suitability of the substrate employed and the conditions of incubation rather than the exact number of viable cells on the material under examination. In dealing with spore-formers the problem becomes further complicated. Viable spores do not necessarily germinate upon the plate although the substrate may be suited to the reproduction of the vegetative cells of the species. Nevertheless, recourse was had to the direct count and likewise to the use of microtome sections in an effort to establish the total number of bacterial cells present on the cured leaf. Such counts were to prove of particular value when studied in connection with direct counts made on the fermenting leaf.

Ground material could not be used for this purpose as the artifacts rendered a count impossible. It was found, however, that if the same procedure was followed as was used in the plating of unground samples a fairly satisfactory count could be made. Such preparations usually showed from 400 million to 800 million cocci and spores per gram of cured tobacco. Rods were seldom seen. It would appear, therefore, that some reproduction must have taken place on the leaf, either during the growing season or during the period of curing.

Contrary to the observations of others, none of the samples examined was found to be sterile. When compared with examinations made of green leaves it was seen that a certain amount of selection had taken place. The flora of the green leaf, including as it does most of the common saprophytes of the soil, had become, in the case of the cured leaf, a flora consisting of cocci, spore-forming bacteria and those fungi commonly termed molds. Some reproduction had apparently taken place; first because of the large number of cocci and spores found by direct observation, and second because of the presence of fungus mycelium upon the leaf.

Flora of the fermenting leaf.—Suchsland *(25)*, Vernhout *(26)*, Schmidt *(23)*, Johnson *(10)* and others have reported the presence

of large numbers of microorganisms upon fermenting cigar-leaf tobacco. These observers have not been in complete accord as to the type or types of microorganisms multiplying during this process. Aerobic spore-forming bacteria, cocci and fungi have each been looked upon as causative agents of the fermentation or aging of this type of tobacco. On the other hand, the proponents of the plant enzyme theory have denied the presence of any considerable number of living microöranisms on the fermenting leaf and have stated that reproduction of such forms was impossible under the conditions of satisfactory fermentation. In an effort to account for these conflicting observations attempts were made in the laboratory to carry out the fermentation of tobacco of various grades over wide ranges of moisture-oxygen-temperature relationships.

It soon became apparent that fungi, cocci and spore-forming bacteria were able to multiply rapidly upon the cured leaf of average quality if proper conditions were provided to satisfy their individual needs. Low moisture and large amounts of oxygen provided suitable conditions for the development of fungi; a slight increase in the moisture content provided a satisfactory environment for the rapid growth of cocci and aerobic spore-formers; anaerobic conditions, brought about in various ways, resulted in the activities of members of *Clostridium* and the anaerobic respiration of the facultative cocci and members of *Bacillus*. Contrary to the observations of Johnson *(10)*, only the aerobic development of cocci and aerobic spore-forming bacteria was found to be associated with a satisfactory fermentation. The judgment of the authors was not relied upon in this connection; samples were submitted to leaf experts in the industry for their decision as to whether or not the fermentation might be termed satisfactory.

Fungi developing upon the cured leaf were usually found to be members of the genera *Aspergillus* and *Penicillium*. Occasionally the development of members of the *Mucorales* was noted upon tobacco of good quality. Growth of the fungi was invariably accompanied by the development of a musty odor.

In the early stages of these investigations it was found that if the environment was suitable for the development of either cocci or spore-forming bacteria the fungi fail to multiply. Throughout the entire course of this study data conflicting with this have never been obtained.

Whether fungi or bacteria develop upon the cured leaf during fermentation is largely controlled by two factors; the composition of the leaf and the amount of moisture present. Data obtained to date do not indicate a definite critical moisture content below which fungi develop and above which bacteria inhibit their devel-

opment that is applicable to all cigar-leaf tobacco. Rather it would appear that the amount of moisture necessary to the development of bacteria is definitely related to the composition of the tobacco or, in terms of the tobacco grower and processor, the quality of the leaf. In general, it has been found that leaf of good quality provides a suitable substrate for bacterial development at lower moisture content than does leaf of poor quality.

Cocci developing upon tobacco undergoing a satisfactory fermentation were found to be members of *Micrococcus* and *Staphylococcus*. Of these the *Micrococci* were usually present in far greater numbers. During later stages of the fermentation, coccoid forms were frequently found in greater numbers. These are thought to be identical with the very short rods described by Schmidt *(23)*. Biochemical studies of these forms have lead to the conclusion that they should be classified with the *Micrococci*.

Aerobic spore-formers associated with the satisfactory fermentation of the leaf have been identified as members of the *subtilis–mesentericus–vulgatus* group of *Bacillus*. These forms are undoubtedly the organisms described by Vernhout *(26)*, to the activities of which he attributed fermentation. They are also the type described by Schmidt *(23)* as associated with the satisfactory fermentation of Ohio tobacco of good quality. For the sake of brevity these will be referred to subsequently as *B. mesentericus*. *B. megatherium* and *B. mycoides* have been observed. These were described by Schmidt *(23)* on fermenting tobacco.

Rapid multiplication of cocci and bacilli upon fermenting tobacco was found to result in the establishment of anaerobic conditions in the center of the fermenting heap. Such conditions resulted in spoilage if allowed to continue. The facultative cocci and bacilli continued to multiply after the exhaustion of the oxygen and, in addition, *Clostrium* sp. became active. Putrid odors developed as the nitrogenous material was attacked anaerobically and eventually the cellulose was attacked and destroyed. Inasmuch as tobacco of good quality favored the most rapid growth of microorganisms, resulting in anaerobiosis in shorter periods than was true with tobacco of poorer quality, this condition was more frequently encountered in connection with the fermentation of the higher quality tobacco. Frequent aeration was found to prevent much of this loss.

Plate counts made during the progress of the fermentation showed that the rapidity of multiplication was more intimately related to moisture content and leaf composition than to other factors. Temperature of course proved of some significance, particularly in dealing with fermenting samples so small that the intense heat generated could not be retained. Temperatures of 35 to 40° C

seemed well adapted to the development of those types associated with a satisfactory fermentation.

In dealing with leaf of at least fair quality held under optimum conditions a rise in plate count from less than 100,000 microörganisms per gram to counts of almost two billion per gram were ordinarily accomplished in less than three days. With the lower moisture content used in case fermentation, such rapid rise was not noted. If the tobacco, however, was undergoing any fermentative changes, a slow rise in plate count could be demonstrated.

The use of the direct count was found to provide much useful information in the study of fermenting tobacco. A rise in numbers during the course of the fermentation was always observed. Direct counts of cured tobacco, as previously stated, usually demonstrated the presence of 500 million to almost one billion spores and cocci per gram of tobacco. The number materially increased as fermentation progressed.

Of equal significance to the demonstration of the rise in numbers was the observation of chains of rods of members of *Bacillus*, conclusive proof of the multiplication of these organisms upon the tobacco. In dealing with spore-formers it is not always possible to be certain that an increase in count by the plate method actually implies multiplication. At times this merely reflects a change in the ability of spores to germinate when placed on the medium. The demonstration of great numbers of chains of the spore-former by the direct method, however, satisfactorily solved the problem as to whether the increased plate count implied reproduction on the leaf or increased germinability of spores already present on the tobacco at the beginning of the fermentation. No chains of spore-forming rods were observed on cured tobacco; apparently spore-formers are present on cured tobacco only in the spore state.

The investigations relative to the flora of fermenting tobacco conducted in this laboratory have shown that fungi, cocci and spore-forming bacteria are able to develop on cured tobacco. They have further shown that a substrate-moisture relationship exists which in large measure determines whether fungi will grow with consequent loss of tobacco through molding; or bacteria may develop with subsequent inhibition of the growth of fungi. These investigations have further demonstrated a substrate-moisture-oxygen relationship to exist which governs the loss of tobacco by rotting.

Relation of the microflora of cigar-leaf tobacco to catalase activity.—Having failed to confirm the findings of Loew *(14)*, in respect to multiplication of microörganisms upon fermenting tobacco, the authors attempted to determine whether a relationship existed between the enzymatic activity of the leaf and the microbial activity observed in this laboratory.

The enzyme catalase was discovered by Loew *(16)* on fermenting tobacco. The use of this enzyme in the present investigation does not imply the belief on the part of the authors that the enzyme is of any particular significance in the tobacco fermentation. The ac-

Table 1.—Effect of five-day storage at 37° C upon the microflora and catalase activity of cured tobacco adjusted to a moisture content of 30 per cent.

Sample Taken	Plate Count of Bacteria per Gram	Ml. N/100 H_2O_2 Decomposed by .2-gram Sample in 60 Minutes
Before storage	52,000	14.2
After 5 days storage	35,000,000	86.9

tivity of catalase is easy to measure, however, and as it was considered of particular significance by Loew it was chosen for use in this work.

Several hundred determinations of catalase activity were made from the tobacco suspensions employed in the routine determinations of bacterial numbers on both cured and fermenting tobacco. A rather close correlation was found between catalase activity and

Table 2.—Effect of bacterial growth on the catalase activity of sterilized cured tobacco, adjusted to a moisture content of 35 per cent and held at 37° C.

Sample Taken	Inoculation	Plate Count of Bacteria per Gram	Ml. N/100 H_2O_2 Decomposed by .2-gram Sample in 60 Minutes
Before storage	None	0	0
Before storage	Culture E-11*	31,000	0
After storage of five days	None	0	0
After storage of five days	Culture E-11	800,000,000	176.2

* Culture E-11 is an isolation made from fermenting tobacco identified with the *subtilis–mesentericus–vulgatus* group of *Bacillus*.

microbial activity. In general any treatment of the tobacco attempted which resulted in inhibition of the bacterial population likewise prevented an increase in catalase activity. Conversely, treatments that gave an increase in bacterial numbers showed an increase in catalase activity. Table 1 shows the typical increase in numbers of bacteria and activity of catalase found in tobacco of av-

erage quality held for a few days under conditions suited to bacterial reproduction.

Heat treatments necessary to render tobacco sterile also rendered it free of catalase activity. It was found, as shown in table 2,

Table 3.—Changes in the microflora of Pennsylvania cigar-leaf tobacco of good quality undergoing a satisfactory case fermentation.

STAGE OF SAMPLING	DATE	PLATE COUNT OF BACTERIA PER GRAM	PLATE COUNT OF FUNGI PER GRAM	PREDOMINANT ORGANISM
At end of curing process	2/27/37	53,000	388,000	*B. megatherium*
After two months fermentation in case	4/26/37	1,840,000	11,000	*B. mesentericus*
After three and one-half months fermentation in case	6/14/37	1,160,000,000	None	Cocci

that normal catalase activity could be imparted to sterilized tobacco by inoculation with organisms previously isolated from fermenting tobacco.

Johnson *(10)* reported that the sterilization of tobacco was accomplished by steaming for one hour, during which time the tobacco reached a temperature of 85° C. Such treatments in this laboratory failed to produce sterility, but intermittent exposure to live steam for 30-minute periods on three successive days proved satis-

Table 4.—Flora of cigar-leaf tobacco of inferior quality that showed unsatisfactory fermentation although cased for a period of four months.

SAMPLE	PLATE COUNT OF BACTERIA PER GRAM	PLATE COUNT OF FUNGI PER GRAM
Sample A	3,500	210,000
Sample B	90,000	45,000

factory. Without employment of the intermittent method of sterilization, an exposure in the autoclave at 15 pounds pressure for a period of 150 minutes was found necessary to insure sterility.

A review of the data obtained in this laboratory leads to the conclusion that the measurements of enzymatic activity of the tobacco leaf made by Loew *(15, 16)* and other investigators of the tobacco fermentation were, after all, merely indirect measurements of microbial activity.

Microflora of case-fermented cigar-leaf tobacco.—Case fermentation of cigar-leaf tobacco as practiced by manufacturers has been studied in these investigations. Typical changes found in satisfactory case fermentation are represented in table 3. The slow rise in bacterial numbers as determined by plate count and the gradual decline in the viability of the fungus spores and mycelium present

Table 5.—A study of the flora during the bulk fermentation of cigar-leaf tobacco which had previously undergone six years of slow fermentation.

Sample	Bulk Temp. °F	pH	Per Cent Moisture	Bacterial Counts per Gram		Plate Count of Fungi per Gram
				Plate *thousands*	Direct *millions*	
As received		6.6	12.8	70	900	1,400
Following water treatment		7.0	61.7	375,000	1,620	300
Following steaming		7.2	61.6	8,500	1,540	1,000
24 Hours after steam treatment		7.2	61.6	3.400	2,600	0
Following stemming		7.4	50.0	40,000	5,900	0
Bulked 36 hours	90	7.6	50.1	1,560,000	7,100	0
Bulked 72 hours	91	7.8	50.5	120,000	7,650	0
Bulked 5 days	92	above 7.8	50.4	312,000	7,100	0
Bulked 7 days	100	above 7.8	50.4	285.000	8.150	0
Bulked 9 days	112	above 7.8	51.3	64,000	7,900	0
Bulked 13 days	125	above 7.8	51.4	5,240	7,800	0
Bulked 17 days	131	above 7.8	51.6	4,350	8,200	0
Bulked 29 days	136 *rebulked*	above 7.8	51.3	520	8,450	0
Bulked 32 days	105	above 7.8	47.1	870,000	10,200	0
Bulked 49 days	136 *rebulked*	above 7.8	43.2	43,000	12,000	0
Bulked 52 days	110	above 7.8	43.0	230,000	14,500	0
Bulked 60 days	136 *rebulked*	above 7.8	42.7	400	14,700	0
Bulked 62 days	115	above 7.8	42.9	32,000	14,600	0
Bulked 69 days	136	above 7.8	40.1	1,130	15,000	0

are characteristic of this practice. It is noted in table 3 that although aerobic spore-formers predominated on the platings at the end of the curing process and after two months fermentation, coccus forms outnumbered bacilli on the plates at the end of three and one-half months. Inasmuch as this tobacco was of the best quality and undergoing the normal fermentation, these findings are at variance with those of Schmidt *(23)*, who concluded that cocci were present in great numbers only on tobacco of inferior quality.

Study was made of a number of tobaccos reported by the manufacturer as failing to ferment in the case. Table 4 presents the data obtained from two such samples that are typical of all received.

The counts presented in this table should be compared with those in table 3 representing determinations made at the end of three and one-half months of case fermentation. Table 3 shows the bacterial plate count on the normally fermenting tobacco to have reached a value in excess of one billion at this stage. On the other hand, table 4 shows that tobacco which has failed to undergo any case fermentation, although held for a period of several months in the case, gives a characteristic bacterial plate count of less than 100,000 organisms per gram. Fungi disappeared in the normally fermenting case at the end of three and one-half months but failed to do so in tobacco that had failed to initiate fermentation.

Microflora of bulk-fermented cigar-leaf tobacco.—Manufacturers of scrap chewing tobacco do not follow the same procedure in the fermentation of cigar-leaf tobacco as do cigar manufacturers. A common practice in the scrap chewing tobacco industry is to allow a slow fermentation of the leaf over a period of two to seven years, followed by a rapid fermentation at high moisture content in bulks containing several tons of tobacco. Bulk fermentation is completed in periods ranging from four to 10 weeks and the tobacco is then ready for manufacture.

Many bulks have been studied; data obtained from one that is characteristic of a satisfactory fermentation are presented in table 5. During the period of slow fermentation the moisture content of the tobacco had been allowed to fall below 15 per cent. At this stage the plate count of bacteria is ordinarily less than 100,000, the colonies on countable plates consisting for the most part of *B. megatherium*. It is significant that the mold count is ordinarily low at this time, much lower than the mold count of the freshly cured leaf.

The first operation in the bulk procedure is the moistening of the tobacco. Warm water is sprinkled on the baled tobacco as received; in this manner the contaminating material is largely washed away. The moistened bales are usually allowed to stand over night. During this time a significant rise in the bacterial count is always noted.

In addition *B. mesentericus* usually becomes the predominant organism at this stage and remains predominant throughout the bulk fermentation in many cases. Although some manufacturers stem the tobacco after the fermentation, the bulks followed in this study were stemmed before bulking. In this case the tobacco was steamed, stemmed and piled into bulks in the course of a few hours. The pH of the tobacco slowly rises during this period, table 5. Although the freshly cured tobacco examined in this laboratory was found to have pH values of 6 to 6.4, the tobacco following the slow fermentation was found to have pH values of 6.4 to 6.8.

Immediately after bulking there is a rapid rise in the bacterial plate count and likewise in the direct count. The latter reveals the

presence of chains of spore-formers in the vegetative state. Cocci were found to predominate at this stage in the bulk from which the data for table 5 were obtained, and this was found to be the case in many bulks, although a number studied showed *B. mesentericus* to be predominant at this stage as well as throughout the remainder of the fermentation. The presence of both organisms in all bulks at all times was easily demonstrated.

The increase in bacteria was accompanied in every instance by the complete disappearance of viable fungi in the bulk and was accompanied by temperature rise and increasing alkalinity.

The bulks studied were turned and in that manner aerated when the temperature in the center of the bulk reached 136° F. This aeration cooled the tobacco to some extent and the cooler, outer leaves were always placed in the center of the new bulk. This aeration was always followed by another rise in the bacterial count which had decreased as the bulk became hot and oxygen supply deficient. Heating following the aeration proved possible as long as sufficient energy material remained to permit bacterial multiplication. From the data obtained in these investigations, it is concluded that the multiplication of certain bacteria on the leaf is intimately associated with the normal fermentation of cigar-leaf tobacco, and that the desired chemical changes are not obtained in the absence of such microbial activity.

SUMMARY

Cured tobacco of the cigar-leaf type has a characteristic microflora in which some selection has already taken place.

Selection continues during fermentation and favored types multiply profusely before the fermentation is completed.

Fungi, which are always present on the cured leaf, are destroyed rapidly in a satisfactory fermentation.

The multiplication of aerobic spore-forming bacteria and cocci characterize the normal fermentation.

Substrate-moisture-oxygen and temperature relationships determine the nature of the microbial activities and consequently the nature of the fermentation.

Unsatisfactory composition of the leaf, termed by the industry "poor quality," favors the multiplication of fungi. A moisture content insufficient for bacterial growth also favors multiplication of the fungi.

Consumption of oxygen by the bacterial population may lead to anaerobic conditions and subsequent spoilage due to anaerobic activities of microörganisms present.

Catalase activity of the leaf is closely related to the activities of the microörganisms present.

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